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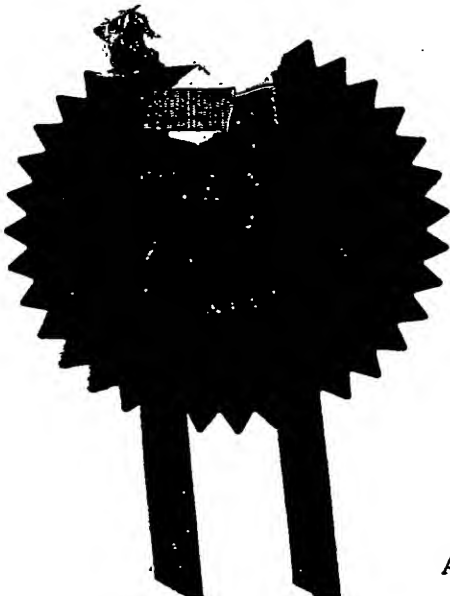
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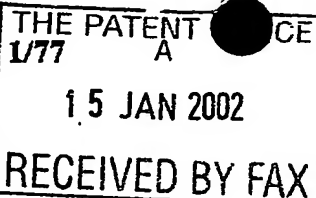
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	15JAN02 E688005-1 D02866		
2. Patent application number (The Patent Office)	0200816.7		
	15 JAN 2002		
3. Full name, address and postcode of the or of each applicant (underline all surnames)	Delta Biotechnology Limited Castle Court 59 Castle Boulevard Nottingham NG7 1FD United Kingdom		
Patents ADP number (if you know it)			
If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom		
4. Title of the invention	CHIRAL SELECTION		
5. Name of your agent (if you have one)	ERIC POTTER CLARKSON PARK VIEW HOUSE 58 THE ROPEWALK NOTTINGHAM NG1 5DD		
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Patents ADP number (if you know it)	1305010		
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application		Date of filing (day / month / year)
8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:	YES		
a) any applicant named in part 3 is not an inventor; or			
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Claims(s) 3 ✓

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11. I/We request the grant of a patent on the basis of this application.

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ERIC POTTER CLARKSON

Date

15 January 2002

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CHIRAL SELECTION

The present invention relates to chiral selection.

Serum albumin has been successfully used as a chiral selector in enantioselective chromatography, such as high-performance liquid chromatography (HPLC). Albumin can act as a chiral stationary phase (CSP) when immobilised, for example, on agarose or silica with, for example, glutaraldehyde (Andersson *et al*, 1992, *J. Chromatogr.*, 591, 65-73). Using such a system, Yang and Hage (1993, *J. Chromatogr.*, 645, 241-250) demonstrated that only the L-enantiomer of tryptophan is bound, whereas Lagercrantz *et al* (1981, *Comp. Biochem. Physiol.*, 69C, 375-378) have used this system to separate chiral forms of warfarin. Racemic mixtures of aryl propionate anti-inflammatory drugs (Noctor *et al*, 1991, *Chromatographia*, 31, 55-69) and N-methylated barbiturates (Krug *et al*, 1994, *Arzneim.-Forsch.*, 44, 109-113) have also been resolved.

The application of serum albumin as a chiral selector has been extended to affinity capillary electrophoresis (Birnbaum and Nilsson, 1992, *Anal. Chem.*, 64, 2872-2874; Arai *et al*, 1994, *Anal. Biochem.*, 217, 7-11. For a review of the use of capillary electrophoresis to separate enantiomers of chiral compounds, see Lloyd *et al* (1997, *J. Chromatogr. A.*, 792(1-2), 349-69).

The serum albumin-based columns used in these methods have had the problem of significant variation of chromatographic performance. The variable efficacy of previously available methods has made the development and validation of HPLC methods difficult.

Modification of methods utilising immobilised albumin as a CSP have usually involved the introduction of additives into the mobile phase. For example, Hayball *et al* (1994, *J. Chromatogr. B*, 662, 128-133) describes

how the separation (*R*)- and (*S*)-enantiomers of ketorolac using human serum albumin (HSA)-CSP can be modified by the addition of octanoic acid into the mobile phase to increase the ratio of percentage unbound (*S*)- to unbound (*R*)-enantiomer. Domenici *et al* (1990, *Chirality*, 2(4), 263-268) reports that the inclusion of bilirubin had no effect on the resolution of enantiomers of tryptophan, but resulted in the loss of resolution of enantiomers of thyronine, whereas the inclusion of caprylate into the mobile phase had the opposite effect. These documents do not provide any clear way to optimise chiral separation using albumin as a CSP. What works for the optimisation of separation of one chiral compound may have the opposite effect on the separation of another chiral compound.

The present invention addresses the problem of variable chromatographic performance.

According to a first aspect of the invention, there is provided the use of highly homogeneous serum albumin as a chiral selector. Preferably the highly homogeneous serum albumin is immobilised.

The term "serum albumin" refers generically to serum-derived albumin and/or recombinantly produced albumin. Preferably, the term "serum albumin" includes within its meaning variant albumin. A "variant albumin" refers to an albumin protein wherein at one or more positions there have been amino acid insertions, deletions, or substitutions, either conservative or non-conservative, provided that such changes result in an albumin protein capable of acting as a chiral selector, as defined below. Typically a variant albumin also substantially retains other basic properties of albumin. For example, a variant albumin may have a binding activity (type of and specific activity), thermostability and/or activity in a certain pH-range (pH-stability) which has not significantly been changed from an albumin protein with the original sequence. "Significantly" in this context means that one skilled in the art

would say that the properties of the variant may still be different but would not be unobvious over the ones of the original protein.

By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr, Lys, Arg; and Phe, Tyr. Such variants may be made using the methods of protein engineering and site-directed mutagenesis known in the art, such as those disclosed in US Patent No 4,302,386 issued 24 November 1981 to Stevens, incorporated herein by reference.

Typically an albumin variant will have more than 40%, usually at least 50%, more typically at least 60%, preferably at least 70%, more preferably at least 80%, yet more preferably at least 90%, even more preferably at least 95%, most preferably at least 98% or more sequence identity with naturally occurring albumin. The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequence has been aligned optimally. The alignment may alternatively be carried out using the Clustal W program (Thompson *et al.*, 1994). The parameters used may be as follows:

Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

Scoring matrix: BLOSUM.

In one preferred embodiment the serum albumin is human serum albumin. The term "human serum albumin" includes the meaning of a serum albumin having an amino acid sequence naturally occurring in humans, and variants

thereof as defined above. Preferably the albumin has the amino acid sequence disclosed in WO 90/13653 or a variant thereof. The term "human serum albumin" also includes the meaning of fragments of full-length human serum albumin or variants thereof. Any fragment may be used, so long as it retains the ability to act as a chiral selector.

In another preferred embodiment the serum albumin is bovine serum albumin. The term "bovine serum albumin" includes the meaning of a serum albumin having an amino acid sequence naturally occurring in cows, and variants thereof as defined above. The term "bovine serum albumin" also includes the meaning of fragments of full-length bovine serum albumin or variants thereof. Any fragment may be used, so long as it retains the ability to act as a chiral selector.

Serum albumin, such as human or bovine serum albumin, for use in the present invention may be serum-derived and is therefore typically obtained by extraction from blood. Examples of extraction and separation techniques include those disclosed in: JP 03/258 728 on the use of a cation exchanger; EP 428 758 on the use of anion exchange; EP 452 753 on the use of heating, adding salt and diafiltering; and WO 96/37515 and WO 00/44772 on complex multi-stage purification processes.

Alternatively the serum albumin used in the present invention is recombinantly produced. Thus a polynucleotide encoding a serum albumin, such as a human or bovine serum albumin, may be transformed into a cell and expressed.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*, *Pichia pastoris* and *Kluyveromyces lactis*), filamentous fungi (for example *Aspergillus*), plant cells, whole plants, animal cells and insect cells.

In one embodiment the preferred host cells are the yeasts *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Pichia pastoris*. It is particularly advantageous to use a yeast deficient in one or more protein mannosyl transferases involved in O-glycosylation of proteins, for instance by
5 disruption of the gene coding sequence.

The albumin protein sequence does not contain any sites for N-linked glycosylation and has not been reported to be modified, in nature, by O-linked glycosylation. However, it has been found that recombinant human serum albumin (rHA) produced in a number of yeast species can be
10 modified by O-linked glycosylation, generally involving mannose. The mannosylated albumin is able to bind to the lectin Concanavalin A. The amount of mannosylated albumin produced by the yeast can be reduced by using a yeast strain deficient in one or more of the *PMT* genes (WO 94/04687). The most convenient way of achieving this is to create a yeast
15 which has a defect in its genome such that a reduced level of one of the *Pmt* proteins is produced. For example, there may be a deletion, insertion or transposition in the coding sequence or the regulatory regions (or in another gene regulating the expression of one of the *PMT* genes) such that little or no *Pmt* protein is produced. Alternatively, the yeast could be transformed
20 to produce an anti-*Pmt* agent, such as an anti-*Pmt* antibody.

If a yeast other than *S. cerevisiae* is used, disruption of one or more of the genes equivalent to the *PMT* genes of *S. cerevisiae* is also beneficial, eg in *Pichia pastoris* or *Kluyveromyces lactis*. The sequence of *PMT1* (or any other *PMT* gene) isolated from *S. cerevisiae* may be used for the
25 identification or disruption of genes encoding similar enzymatic activities in other fungal species. The cloning of the *PMT1* homologue of *Kluyveromyces lactis* is described in WO 94/04687.

The yeast will advantageously have a deletion of the *HSP150* and/or *YAP3* genes as taught respectively in WO 95/33833 and WO 95/23857.

In a preferred embodiment the yeast is transformed with an expression plasmid based on the *Saccharomyces cerevisiae* 2 μ m plasmid. At the time
5 of transforming the yeast, the plasmid contains bacterial replication and selection sequences, which are excised, following transformation, by an internal recombination event in accordance with the teaching of EP 286 424. The plasmid also contains an expression cassette comprising: a yeast promoter (eg the *Saccharomyces cerevisiae* *PRB1* promoter), as taught in
10 EP 431 880; a sequence encoding a secretion leader which, for example, comprises most of the natural HSA secretion leader, plus a small portion of the *S. cerevisiae* α -mating factor secretion leader as taught in WO 90/01063; the HSA coding sequence, obtainable by known methods for isolating cDNA corresponding to human genes, and also disclosed in, for
15 example, EP 73 646 and EP 286 424; and a transcription terminator, preferably the terminator from *Saccharomyces ADHI1*, as taught in EP 60 057. Preferably, the vector incorporates at least two translation stop codons.

The choice of various elements of the plasmid described above is not thought to be directly relevant to the purity of the albumin product obtained,
20 although the elements may contribute to an improved yield of product.

Techniques for the purification of serum and recombinantly expressed albumin are well known in the art.

An impure albumin solution may, for example, be obtained from serum by any of the plethora of extraction and purification techniques developed over
25 the last 50 years, for example those disclosed in Stoltz *et al* (1991) *Pharmaceut. Tech. Int.* June 1991, 60-65 and More & Harvey (1991) in "Blood Separation and Plasma Fractionation" Ed. Harris, Wiley-Liss, 261-

306. Alternatively an impure albumin solution maybe obtained from the fermentation medium for a micro-organism transformed with a nucleotide sequence encoding the amino acid sequence of albumin; preferably separating the micro-organism from the fermentation medium; and
5 conditioning the medium, if necessary, for further purification. In a further alternative, an impure albumin solution may be obtained, for example, from a transgenic animal, such as goat, sheep or cattle, from, for instance, the milk or the blood of the animal or, in the case of transgenic chicken, from the egg white. In a still further alternative, an impure albumin solution may
10 be obtained from a transgenic plant, such as tobacco, potato or corn (maize).

Typically the process of obtaining highly pure albumin from an impure albumin solution comprises one or more of the following steps: exposing the impure albumin solution to three successive chromatography steps; ultrafiltering/diafiltering the product; passing the ultrafiltered product
15 through a further chromatography step; ultrafiltering/diafiltering again before purification through two further chromatographic steps; and final ultrafiltration/diafiltration.

Preceding or following any of the above mentioned procedures the albumin solution may undergo buffer exchange, concentration, dilution, heating
20 (including sterilisation), cooling or may have salts etc. added to the albumin solution which may, for example, condition or adjust the pH of the solution. Optionally, the albumin may be treated with a reducing agent or may undergo a decolouration step.

The final product may be formulated to give it added stability. Any
25 stabilising agent known in the art may be used. Typically, octanoate or N-acetyl tryptophan may be used as a stabilising agent.

Where a serum albumin is formulated with a stabilising agent, it is preferably subjected to defatting prior to its use according to the first aspect

of the present invention. Any suitable process known in the art, such as Chen's charcoal process (Chen RF, 1967, *J. Biol. Chem.*, 242, 173-181), may be used to defat a serum albumin formulated with a stabilising agent.

For an example of the purification of recombinantly expressed albumin, see
5 EP 658 569, or preferably WO 96/37515 and WO 00/44772.

Using such methods it is possible to obtain highly homogenous serum albumin. Highly homogeneous albumin can be characterised by a number parameters. Highly homogeneous albumin will have at least one property selected from the group consisting of:

- 10 (i) extremely low levels of colorants. The term "colorant" as used herein means any compound which colours albumin. For example, a pigment is a colorant which arises from the organism, such as yeast, which is used to prepare recombinant albumin, whereas a dye is a colorant which arises from chromatographic steps to purify the albumin.
- 15 (ii) extremely low levels of, or be essentially free of, aluminium, lactate, citrate, metals, non-albumin human proteins, such as immunoglobulins, prekallikrein activator, transferrin, α_1 -acid glycoprotein, haemoglobin and blood clotting factors, prokaryotic proteins, fragments of albumin, albumin aggregates or polymers, or endotoxin, bilirubin, haem, yeast proteins,
20 animal proteins and viruses. By essentially free is meant below detectable levels.
- (iii) at least 99.5% monomeric and dimeric, preferably essentially 100% monomeric and dimeric. Up to 0.5%, preferably 0.2% trimer is tolerable but larger forms of albumin are generally absent.
- 25 (iv) a nickel ion level of less than 100ng, based on one gram of albumin.

- (v) a glycation level of less than 0.6, preferably less than 0.10, 0.075 or 0.05 moles hexose/mole protein as measured in the Amadori product assay.
- (vi) at least 90% or 95%, preferably at least 96%, more preferably at least 97%, even more preferably at least 98%, even more preferably at least 99%,
5 most preferably substantially 100% of the albumin molecules have an intact C-terminus.
- (vii) a content of conA-binding albumin of less than 0.5% (w/w), preferably less than 0.2% or 0.15%.
- (viii) a free thiol content of at least 0.85 mole SH/mole protein.
- 10 (ix) substantially no C18 or C20 fatty acids.

The homogeneity of population of serum albumin molecules, may be analysed by SDS PAGE, native PAGE and gel permeation chromatography. Usually, when analysed by SDS PAGE native PAGE and gel permeation chromatography, a highly homogeneous serum albumin preparation will
15 display at least one, preferably two, of the following features:

- (a) at least 99%, preferably 99.9% of the protein molecules in the population will be serum albumin.
- (b) no more than 2%, preferably no more than 1% of albumin protein molecules in the population will dimeric.
- 20 The homogeneity of population of serum albumin molecules, may also be analysed by electrospray mass spectrometry (EMS) and by peptide mapping.

In a preferred embodiment, when analysed by ESMS and peptide mapping, a highly homogeneous serum albumin preparation will have the correct

native primary sequence of full length HSA or a fragment thereof as defined above and will not have post-translational modifications.

Albumin may be immobilised on any suitable support. Preferably, a silica matrix is used to immobilise the serum albumin. Alternatively, serum
5 albumin can be immobilised on agarose or a polymer matrix, such as polystyrene divinyl benzene (PSDB).

Albumin can be immobilised by methods well known in the art. For example, methods for protein immobilisation are reviewed in Haginaka (2001, *J. Chromatogr. A*, 906, 253-273), the teachings of which are
10 incorporated herein by reference.

Serum albumin can be immobilised on silica gel, for example, by following the protocol provided in the examples.

Serum albumin can be immobilised on agarose activated with cyanogen bromide. 200mg of CNBr/ml agarose will couple 5-10 mg/ml of albumin.

15 Albumin is "enantioselective" because it is able to bind with greater affinity to one enantiomer of at least some chiral compounds than to the other enantiomer. Typically, albumin, when exposed to a mixture of enantiomers of a chiral compound for a period to allow selective binding of one enantiomer to the albumin, will bind to one of the enantiomers with an
20 affinity that is up to 10^1 - fold, 10^2 - fold, 10^3 - fold, 10^4 - fold, 10^5 -fold, 10^6 - fold, or more, greater than the affinity with which the other enantiomer is bound.

The term "mixture of enantiomers" typically refers to a racemic mixture, although other proportions, such as about 99:1, 95:5, 90:10, 80:20, 70:30,
25 60:40, 55:45 and 51:49, as well as all proportions in between these values, are included in the meaning.

Albumin, when exposed to a mixture of enantiomers of a chiral compound, will selectively bind to one enantiomer under suitable conditions. The phrase "suitable conditions" includes in its meaning all conditions at which one enantiomer of the chiral compound has a higher binding affinity for the highly homogenous serum albumin than the other enantiomer. These conditions are well known to the skilled person and optimal conditions can be established for each different chiral compound by routine testing. Typically, suitable conditions can be achieved by using a suitable buffer, such as 1-propanol/phosphate buffer 15/85 (v/v), at a suitable pH, such as between pH 6-9, preferably at about pH 7.5.

As a non-limiting example, enantiomer separation can be performed by HPLC using highly homogeneous serum albumin immobilised on silica gel and a 1-propanol/phosphate buffer (pH 7.5) 15/85 (v/v) with a 0.6 ml/min flow rate.

The highly homogeneous serum albumin according to the first aspect of the invention can be used to separate enantiomers of any chiral compound that interact differentially with albumin. This can be readily tested, for example, by using circular dichroism to determine stereospecific binding of a chiral compound to albumin as described in the examples. However, highly homogeneous serum albumin will typically be useful to separate enantiomers of chiral compounds such as amino acids, non-steroidal anti-inflammatory drugs, aryl propionate anti-inflammatory drugs and N-methylated barbiturates. In particular, highly homogeneous serum albumin will typically be useful to separate enantiomers of chiral compounds such as warfarin, lorazepam hemisuccinate, N-benzoyl-DL-leucine, amino acids such as L- and D-tryptophan and non-steroidal anti-inflammatories such as ibuprofen. For the avoidance of doubt, a chiral compound, whose enantiomers are suitable for separation according to the present invention,

may comprise more than one chiral centre and may, for example, be a diastereomer.

Thus highly homogeneous serum albumin as described above can be used as a chiral selector. Typically this involves the use of the serum albumin, 5 immobilised to a solid support, in enantioselective chromatography. However, other suitable techniques include capillary electrophoresis (CE).

In a preferred embodiment of the first aspect of the invention, the type of chromatography for which immobilised highly homogeneous serum albumin is used is high performance liquid-phase (HPLC) chromatography, 10 although the immobilised highly homogeneous serum albumin can also be used in other forms of chromatography, such a 'normal pressure' liquid chromatography.

Where highly homogeneous serum albumin is used in HPLC, it is particularly preferred if the highly homogeneous serum albumin is 15 immobilised on a silica matrix.

Accordingly, a second aspect of the present invention provides an enantioselective chromatography column comprising, as the immobilised phase, highly homogeneous serum albumin. The column is preferably suitable for use in HPLC. Although any matrix to which albumin can be 20 immobilised may be used, typically the column comprises an agarose matrix, a polymer (such as PSDB) matrix or, preferably, a silica matrix, as an immobile phase to which the highly homogeneous serum albumin is affixed.

In a third aspect of the present invention, there is provided a process for 25 selecting an enantiomer of a chiral compound, comprising the steps of

(i) exposing a mixture of enantiomers of the compound to highly homogenous serum albumin for a period to allow selective binding of one enantiomer to the albumin; and

(ii) separating the relatively unbound enantiomer from the albumin.

5 Preferably the highly homogeneous serum albumin used in a process according to the third aspect of the invention is immobilised. Even more preferably the highly homogeneous serum albumin used in a process according to the third aspect of the invention is immobilised in a column according to the second aspect of the invention. Yet more preferably the
10 highly homogeneous serum albumin used in a process according to the third aspect of the invention is immobilised to a column suitable for use in HPLC.

Accordingly, using a process according to the third aspect of the invention, the skilled person can separate enantiomers of a chiral compound wherein
15 those enantiomers interact differentially with albumin, such as those described above.

Thus in one embodiment the thus separated (relatively unbound) enantiomer is the enantiomer of interest.

In another embodiment, the enantiomer relatively bound by the albumin is
20 the enantiomer of interest. In that case, a process of the third aspect of the invention preferably further comprises the step of separating the relatively bound enantiomer from the albumin.

Typically this further step is achieved by eluting the absorbed the relatively bound enantiomer from the albumin using techniques well known in the art.
25 For example, elution of the relatively bound enantiomer can be achieved by varying the environment of the highly homogeneous albumin by one or more parameter selected from ionic strength, pH, temperature, or the

concentration of a displacing ligand (a ligand, different to the two enantiomers being separated, which competes for binding to the albumin). Elution of the relatively bound enantiomer may also be achieved using organic solvent modification of the albumin.

- 5 In either case, the enantiomer of interest can be obtained in a form that is enriched in one enantiomer, relative to the abundance of that enantiomer in the starting material.

Usually, where a substantially racemic mixture is separated, one enantiomer will represent at least 60% of the separated chiral compound obtained by the method of the third aspect of the invention. Typically, one enantiomer will represent at least 70%, more typically at least 80%, even more typically at least 90% of the separated chiral compound obtained by the method of the third aspect of the invention. In a preferred embodiment, one enantiomer will represent at least 95%, more preferably at least 98%, even more preferably at least 99%, yet more preferably at least 99.9%, most preferably, substantially 100% of the separated chiral compound obtained by the method of the third aspect of the invention.

If the mixture of enantiomers to be separated by the method of the third aspect of the invention is not substantially racemic, for example if the enantiomer of interest is represented at, say, less than 25%, 10%, 5%, 2% or 1% of the total chiral compound to be separated, then a process according to the third aspect of the invention may nevertheless be used to increase the relative abundance of the enantiomer of interest in the product of the process. Thus, where the mixture of enantiomers to be separated by the method of the third aspect of the invention is not substantially racemic, the enantiomer of interest may represent at least 1%, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, at

least 99.5%, at least 99.9% or substantially 100% of the separated chiral compound obtained by the method of the third aspect of the invention.

Once an enantiomer has been obtained in an enriched form using a method according to the third aspect of the invention, that enantiomer can be
5 formulated with a pharmaceutically acceptable carrier or diluent.

A pharmaceutically acceptable carrier or diluent must be "acceptable" in the sense of being compatible with the enantiomer and not deleterious to the recipients thereof. Typically, the carrier or diluent will be water or saline which will be sterile and pyrogen free.

- 10 An enantiomer formulated with pharmaceutically acceptable carrier or diluent can be presented in a unit dosage form. For example, the formulation may be presented in a unit dosage form suitable for administration by any conventional method including oral and parenteral (eg subcutaneous or intramuscular) injection.
- 15 Formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (compound of the invention) with the carrier, which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly
20 and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention may be suitable for oral administration and may be presented as discrete units such as capsules,
25 cachets or tablets, each containing a predetermined amount of the enantiomer; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil

liquid emulsion. The enantiomer may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by
5 compressing in a suitable machine the enantiomer in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent.
10 Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired
15 release profile.

Formulations suitable for topical administration in the mouth include lozenges comprising the enantiomer in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the enantiomer in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the
20 enantiomer in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions
25 which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier,

for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit,
5 daily sub-dose or an appropriate fraction thereof, of an enantiomer.

It should be understood that in addition to the ingredients particularly mentioned above the formulations that may be produced using the methods according to the third aspect of the invention may include other agents conventional in the art having regard to the type of formulation in question,
10 for example those suitable for oral administration may include flavouring agents.

In a fourth aspect of the present invention, there is provided an enantiomer of a chiral compound obtainable by a method according to the third aspect of the invention.

15 The invention will now be described in more detail by reference to the following Examples.

Examples

Purification of Recombinant Human Serum Albumin (rHA)

The rHA sample, obtained by expression of HSA gene in the yeast
20 *Saccharomyces Cerevisiae*, was supplied by Delta Biotechnology Limited (Castle Court, Nottingham, UK) under the name "RECOMBUMIN 20" in aqueous solution 25% (w/v) (145 mmol/L sodium chloride, 0.16 mmol sodium octanoate/g albumin and 15 mg/L Tween 80). The protein was defatted before use by a slightly modified version of Chen's charcoal
25 procedure (Chen RF, 1967, *J. Biol. Chem.*, 242, 173-181). The sample of rHA was dialysed against a KH_2PO_4 10 mM/1-propanol 80/20 (v/v)

mixture, with 0.5 g charcoal/g rHA, for 1 hour. The pH of the suspension was adjusted at pH 3 with HCl 0.1 N. Eight subsequent washings were performed decreasing progressively the propanol concentration from 20% to 1%. The last four dialysis processes were performed against phosphate
5 buffer 10 mM at pH 3, 4.6, 7.4 and 7.4, respectively.

The success of the purification was checked by determining the binding properties of the protein for ligands that selectively bind at specific binding sites. In particular phenylbutazone, diazepam and bilirubin were used as markers. These compounds are known to bind selectively at site I, site II and site III
10 on human serum albumin. The induced CD signal observed in the ligand binding to albumin was measured as difference between the spectrum of the drug-protein complex and the spectrum of the protein. The CD difference spectra were carried out in the region where the lowest energy electronic transition of the ligands occurs. This allowed analysis of the CD data at a
15 wavelength where the albumin gives a negligible contribution, as in the case of phenylbutazone and diazepam, or it does not contribute at all, as in the case of bilirubin. The measurement of the CD difference spectra gives direct information on the enantioselectivity of the binding between the ligand and the protein, the observed CD signal being directly related to the amount of
20 bound ligand.

Thus CD difference data were used to determine the binding parameters. In particular, the affinity constants of the drugs, at the high affinity binding sites, were determined by following the change of the intensity of the induced CD spectra upon the molar concentration of the 1/1 [drug/albumin]
25 adduct. CD technique monitors only enantioselective binding. In the case of the used markers the high affinity binding sites are enantioselective. We used progressive dilutions of the [rHA]/ [drug] adduct in a 1/1 molar ratio. The intensity of the induced CD spectra decreases by decreasing the concentration of the complex, depending on the value of the dissociation

constant. The K values obtained for the recombinant and the serum-derived human serum albumins are, for the high affinity binding sites, quite close for all the three markers.

Secondary Structure of rHA

- 5 The purified sample of rHA showed a secondary structure comparable with that of serum-derived human serum albumin, as evaluated by CD. Indeed the CD of the two proteins is almost superimposable in the high-energy portion of the spectrum (260-180 nm).

Immobilization Of The Protein On The Silica Matrix

- 10 Silica gel was heated for 15 h at 180 °C and 1 mm Hg. 2 g of distilled (3-glycidoxypopyl)trimethoxysilane in 5 ml of dry xylene were added drop wise to 5.2 g of silica in 100 ml of dry xylene, and the mixture was refluxed for 36 h. After filtration on a fritted disk of porosity 4, the product was washed twice with dry xylene and dry acetone. The bonded silica was dried
15 for 18 h at 40 °C under vacuum. The activated silica was then packed into 150x4.6 mm I.D. HPLC columns. The column was washed with 100 ml of acetone and dried at 70 °C under helium. A solution of the protein (10 mg/ml) in potassium phosphate buffer (0.05 M, pH 7.5) containing 2 M ammonium sulphate was circulated through the column, in closed circuit for
20 24 h. The column was then rinsed with 100 ml of 0.05 M KH_2PO_4 (pH 6.0) solution. After that, the column was flushed with 50 ml of 1 M glycine in 0.05 M KH_2PO_4 (pH 7.0) solution and then rinsed with 20 ml of 0.05 M KH_2PO_4 (pH 7.0) solution.

- Before storage, 100 ml of potassium phosphate buffer (0.05 M, pH 7.5)
25 solution containing 0.01% sodium azide was passed through the column. The amount of the immobilised protein was determined by UV absorbance

at 280 nm of the protein solution before and after the immobilisation procedure.

Immobilisation involves a reaction between an amino group of a lysine residue of the protein and the epoxide groups of the derivatized silica.

5 Enantioselective HPLC Analysis by the rHA-Base Column

The rHA-based column resulted efficient in the resolution of a variety of chiral drugs and amino acid derivatives. As an example enantiomeric resolution was obtained for *rac*-warfarin ($\alpha = 2.1$), *rac*-lorazepam hemisuccinate ($\alpha = 5.3$), N-benzoyl-DL-leucine ($\alpha = 2.8$), using 1-
10 propanol/phosphate buffer (pH 7.5) 15/85 (v/v), 0.6 ml/min flow rate. The obtained values of enantioselectivity are comparable or higher with respect to those obtained with the corresponding HSA-based columns, under the same experimental conditions.

CLAIMS

1. Use of highly homogeneous serum albumin as a chiral selector.
2. The use according to Claim 1 wherein the highly homogeneous serum albumin is immobilised.
- 5 3. Use of immobilised highly homogeneous serum albumin according to Claim 2 in enantioselective chromatography.
4. Use according to Claim 3 wherein the chromatography is high performance liquid (HPLC) chromatography.
- 10 5. Use according to any one of the preceding claims wherein the immobilised highly homogeneous serum albumin is immobilised on a silica matrix.
6. Use according to any one of the preceding claims to select an enantiomer of a chiral compound selected from an amino acid, a non-steroidal anti-inflammatory drug, an aryl propionate anti-inflammatory
15 drug or an N-methylated barbiturate.
7. Use according to any one of the preceding claims to select an enantiomer of a chiral compound selected from warfarin, lorazepam hemisuccinate, N-benzoyl-DL-leucine, tryptophan or ibuprofen.
- 20 8. An enantioselective chromatography column comprising, as the immobilised phase, highly homogeneous serum albumin.
9. An enantioselective chromatography column according to Claim 8 for use in HPLC.

10. An enantioselective chromatography column according to Claim 8 or 9 wherein the highly homogeneous serum albumin is immobilised on a silica matrix.
11. A process for selecting an enantiomer of a chiral compound, comprising
5 the steps of
- (i) exposing a mixture of enantiomers of the compound to highly homogenous serum albumin for a period to allow selective binding of one enantiomer to the albumin; and
- (ii) separating the relatively unbound enantiomer from the albumin.
- 10 12. A process according to Claim 11 wherein the highly homogenous serum albumin is immobilised.
13. A process according to Claim 12 wherein the immobilised highly homogeneous serum albumin is immobilised on an enantioselective chromatography column as defined in any one of Claims 8 to 10.
- 15 14. A process according to any one of Claims 11 to 13 further comprising the step of separating the relatively bound enantiomer from the albumin.
15. A process according to any one of Claims 11 to 14 wherein the chiral compound is selected from an amino acid, a non-steroidal anti-inflammatory drug, an aryl propionate anti-inflammatory drug and an N-methylated barbiturate.
20
16. A process according to any one of Claims 11 to 15 wherein the chiral compound is selected from warfarin, lorazepam hemisuccinate, N-benzoyl-DL-leucine, tryptophan and ibuprofen.

17. A process according to any one of Claims 11 to 16 further comprising the step of formulating the thus separated enantiomer with a pharmaceutically acceptable carrier or diluent.
18. A process according to Claim 17 further comprising the step of
5 presenting the thus formulated enantiomer in a unit dosage form.
19. A use, an enantioselective chromatography column, or a process according to any one of the preceding claims wherein the highly homogeneous serum albumin is highly homogeneous human serum albumin.
- 10 20. A use, an enantioselective chromatography column, or a process according to any one of the preceding claims wherein the highly homogeneous serum albumin is highly homogeneous recombinant serum albumin.
21. An enantiomer of a chiral compound obtainable by a method according
15 to any one of Claim 11 to 16.
22. A use, an enantioselective chromatography column, or a process
substantially as described herein with reference to the examples. 1

ABSTRACT

CHIRAL SELECTION

The present invention provides the use of highly homogeneous serum albumin as a chiral selector, preferably immobilised, such as in
5 enantioselective chromatography. The present invention also provides an enantioselective chromatography column comprising, as the immobilised phase, highly homogeneous serum albumin. The present invention also provides a process for selecting an enantiomer of a chiral compound, comprising the steps of exposing a mixture of enantiomers of the compound
10 to highly homogenous serum albumin for a period to allow selective binding of one enantiomer to the albumin; and separating the relatively unbound enantiomer from the albumin.